United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

Polymerase Chain Reaction Assay for Detection and Identity of Extraneous Reticuloendotheliosis Virus (REV)

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1. Introduction

This Testing Protocol (PRO) describes a polymerase chain reaction (PCR) assay for detection and identity of extraneous reticuloendotheliosis virus (REV) in biologic products as specified in the guidelines of Veterinary Services Memorandum Number 800.88.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Laminar Flow Biological Safety Cabinet (BSC) (NuAire Inc., Labgard)

Note: Use good laboratory practices to perform the DNA template preparation of the positive controls in an area not related to the testing of other poultry viruses.

2.1.2 Thermocycler (Applied Biosystems, GeneAmp PCR System 9700 or Veriti 96-well thermocycler)

2.1.3 Thermo EC Electrophoretic gel system

2.1.4 Eppendorf Adjustable Volume Research micropipettes, 2.5-µL, 10-µL, 20-µL, 100-µL, 200-µL, 1000-µL

Note: Separate Micropipettes are assigned to the individual BSCs and should not be interchanged between designated hoods to avoid contamination.

2.1.5 Pipette-aid automatic pipettor (Drummond Scientific)

2.1.6 Microcentrifuge (Eppendorf 5415 C)

2.1.7 Heating Block or Water Bath set at 56°± 2°C

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Current versions of the following Virology Testing Worksheets: **VIRTWS0119**, **VIRTWS0219**, **VIRTWS0319**, **VIRTWS0419**, **VIRTWS0519**

2.2.2 DNeasy Blood and Tissue Kit (Qiagen, Catalog #6504)

2.2.3 REV positive control

2.2.4 AmpliTaq Gold with GeneAmp, containing AmpliTaq Gold, 10x PCR Gold Buffer, and $MgCl_2$ (Applied Biosystems, Part #4311806)

2.2.5 GeneAmp dNTP Mix with dTTPs 10mM (2.5mM each) (Applied Biosystems, Product #N8080260)

2.2.6 REV primers, rev-1 and rev-2 at approximately 50 pmol/µL each

Reconstitution of the primers is determined by the synthesis data sheet. The primer batch yield is recorded on the sheet. Convert the nmol amount to pmol by multiplying the amount in nmols by 1000 and dividing the result by 50 to determine amount in microliters of water (PCR grade) to add for a final concentration of 50 pmol per 1 µL.

Primer Sequences:

rev-1, 5'- CATACTGGAGCCAATGGTT -3'

rev-2, 5'- AATGTTGTACCGAAGTACT -3'

2.2.7 PBS for HI (National Centers for Animal Health [NCAH], Media #30102)

2.2.8 Ethanol 200 proof molecular grade (Sigma-Aldrich, Product #E7023)

2.2.9 Agarose 3:1 High Resolution Blend (Amresco, Catalog #E776) which is optimized for molecular weights 100-1000 bp

2.2.10 PCRSizer 100 base pair (bp) ladder (Norgen, Catalog #11400)

2.2.11 Loading Buffer, 10X BlueJuice (Invitrogen, Catalog #10816-015)

2.2.12 Tris Borate EDTA (TBE), 10X (NCAH Media #30387)

2.2.13 Ethidium Bromide (EtBr), 10mg/ml (Invitrogen, Catalog #15585-011)

Caution: Ethidium bromide is a suspected carcinogen. Use gloves when handling any reagents containing Ethidium Bromide. Refer to Material Safety Data Sheet (MSDS) and the NCAH Safety and Security Unit guidelines for safe handling, usage, and disposal.

2.2.14 PCR reaction tubes (Applied Biosystems, MicroAmp, Part #N8010580)

2.2.15 PCR reaction tube caps (Applied Biosystems, MicroAmp, Part #N8010535)

2.2.16 96-well PCR reaction tube tray/retainer (Applied Biosystems, MicroAmp, Part #N8015530)

2.2.17 96-well PCR reaction tube base (Applied Biosystems, MicroAmp, Part #N8015531)

2.2.18 Cap Installing Tool (Applied Biosystems, Part #N8010438)

2.2.19 Plastic 96-well Microtiter Plate (Dynatech Laboratories, Catalog #001-010-2801)

2.2.20 Aerosol Resistant Filter tips for micropipettes, various sizes

2.2.21 Screw Cap Micro Tubes (Sarstedt, Catalog #72.694.006)

2.2.22 DNase/RNase Free water appropriate for use in PCR master mix

2.2.23 Latex, vinyl, or nitrile powder-free disposable gloves

2.2.24 1.5-mL centrifuge tubes

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation and the proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturers' instructions and monitor for compliance with current corresponding standard operating policies/procedures (SOPs). Wear non-powdered disposable gloves. Program the thermocycler (see thermocycler manual) with the following steps:

Amplification of REV DNA

Pre-PCR Heat Start Hold: 5 minutes at 95°C

Cycles 1 - 35: 30 seconds at 95°C
45 seconds at 47°C
1 minute at 72°C, with a 1 second increase following each cycle
Post-PCR Hold: 10 minutes at 72°C
Soak/Storage: 4°C

3.3 Preparation of reagents/control procedures

3.3.1 The REV positive control is Qiagen spin column purified on a separate occasion to avoid cross contamination. See **Section 4.1** for DNA extraction. Keep extracted REV positive control frozen until ready to use. **Once thawed, refrigerate.**

3.3.2 Prepare 1X TBE from 10X TBE using distilled or deionized water. If using as gel buffer, add 0.2 µL Ethidium Bromide to 1 mL 1X TBE.

3.4 Preparation of the sample

Sample is passaged through chicken embryo fibroblast cell cultures according to the current versions of **VIRSOP0013** and **VIRSOP0014** and harvested after the third and final passage. Records of cell passage are kept with extraneous avian leukosis virus (ALV) testing records. Qiagen purify 200 µL of the final cell passage of sample and a negative cell control. See **Section 4.1** for DNA extraction. Keep extracted samples frozen until ready to use.

Note: REV is an RNA virus, and this PCR process is NOT reverse transcriptase PCR. The passages in cells are carried out to increase the chances of detecting extraneous viral cDNA, which is created during the reverse transcription of the RNA virus.

4. Performance of the Test

4.1 DNA extraction

It is recommended that the sample preparation with the positive REV control be prepared in a separate area than the amplification step to avoid cross contamination. Wear disposable, powder-free vinyl, nitrile, or latex gloves for all portions of the test procedure.

4.1.1 Record sample information on the current version of **VIRTWS0119**.

4.1.2 Transfer 200 µL of the sample to a 1.5-mL centrifuge tube.

4.1.3 From this point, follow the protocol that is provided in the Qiagen DNeasy Blood and Tissue Kit, starting with the addition of 20 µL of proteinase K to the sample, and continue from there.

4.1.4 Complete the process for the 200 µL of negative control as well.

4.1.5 Once the Qiagen purification and extraction is completed, store the tubes at -20°C until ready to continue to **Section 4.2.**

4.2 Amplification of REV

4.2.1 See the current version of **VIRTWS0219** for the master mix worksheet**.** Prepare master mix for DNA amplification of REV as follows:

1. Calculate amount of master mix needed by determining total number of tubes (reactions) needed plus one extra.

Example:

See **Section 4.2.2** for sample tray layout. In the sample tray layout, each sample would have 1 reaction with just sample and one reaction of sample with 1 µL of REV control as an internal positive control. For three samples, that would give a total number of six reactions. There is also one tube for an external positive control and one tube for a negative control giving another two reactions. The total number of reactions then is eight, plus one extra reaction. The amount of each ingredient listed below would then be multiplied by nine, which will give the amount of each component required in the master mix.

2. Add the master mix ingredients in the order listed in the following section (amounts are for 1 reaction) in the clean master mix area.
Note: Add Taq polymerase last to the master mix.

Master Mix	100 µL rxn	50 µL rxn*
DNase/RNase free water	66.5 µL	33.0 µL
10X buffer	10.0 µL	5.0 µL
dNTPs	10.0 µL	5.0 µL
$MgCl_2$	8.0 µL	4.0 µL
primer cav-1 50 pmol/µL	2.0 µL	1.0 µL
primer cav-2 50 pmol/µL	2.0 µL	1.0 µL
Taq Polymerase	0.5 µL	0.5 µL
Total Volume per reaction	99.0 µL	49.5 µL

*The Veriti thermocycler has a 50 µL reaction volume.

4.2.2 The following figure shows an example PCR tray layout.

		1	2	3	4
	A	Sample 1	Sample 2	Sample 3	Negative
1 µL REV positive control	**B**	↓	↓	↓	↓

Insert PCR reaction tube strips into two rows on the tray/retainer assembly. Snap top of assembly to secure strips. Place assembly on base.

4.2.3 In the template BSC, add 1 µL** of sample template to a PCR reaction tube in Rows A and B. Repeat this process for each sample. In the last column, add 1 µL of water to a PCR reaction tube in Rows A and B.

****A 10X concentration (10 µL) of sample can be included if low levels of REV are suspected, such as for a REV contamination investigation.**

4.2.4 Transfer 99 µL (90 µL for a 10x concentration of sample) or 49.5 µL of the master mix to each reaction tube in Row A, making sure to use a different pipette tip for each tube master mix is added to. Ensure that the contents of each tube are thoroughly mixed by pipetting mixture up and down several times. In Row A, the tubes with sample template added are the test samples. The tube with water added is the negative control.

4.2.5 Cap all the tubes in Row A. If necessary, use a capping instrument.

4.2.6 To Row B, add 1 µL of REV positive control to each tube, making sure to use a different pipette tip for each sample. After the positive control is added, add 99 µL (90 µL for a 10x concentration of sample) or 49.5 µL of master mix to each tube, once again making sure to use a different pipette tip for each sample. Ensure that the contents of each tube are thoroughly mixed by pipetting mixture up and down several times. The tubes in Row B with sample template and positive control are the internal positive controls. The tube with extracted negative cell control and positive control is the external positive control.

4.2.7 Cap all the reaction tubes in Row B with caps, using a capping instrument if necessary.

4.2.8 Record the tray set up in the PCR sample identification area on the second page of **VIRTWS0119**. The total reaction volume will be 100 µL or 50 µL. (The additional 1 µL volume of positive control is negligible.)

4.2.9 Place the tube holder in the thermocycler and close the heated cover.

4.2.10 Run the REV amplification program (see **Section 3.2**). Make sure the reaction volume is set at 100 µL for the 9700 thermocycler and 50 µL for the Veriti thermocycler.

4.2.11 Once the program is completed, store the tubes at 4°C until ready to analyze PCR product(s).

4.3 Analysis of amplified REV DNA

Wear disposable, powder-free nitrile, vinyl, or latex gloves for all portions of analysis.

4.3.1 Loading gel. See the current version of **VIRTWS0319** for the gel worksheet.

1. Make sure to set up the electrophoresis apparatus before heating the gel, including setting up the tray and adding the comb. Make sure the apparatus is level and the comb is not hitting the bottom of the tray. Comb sizes range from 8 to 12 wells for the small gel and 22 to 40 wells for the large gel. The volume of the wells ranges from 10-20 µL.

2. The gel used is a 2% 3:1 agarose gel. Gel size is determined by the number of lanes needed for the batch. Weigh agarose for appropriate gel size (example: 0.7 g per 35 mL - small gel, 2 g per 100 mL – large gel).

The 1X TBE buffer **without EtBr** is used as a diluent for the 2% gel. EtBr is not added until after heating to avoid dissipation of the EtBr fumes. Combine buffer and powdered agarose in either a screw top glass flask or bottle. Make sure to leave the lid loose. Heat the solution in the microwave until the liquid is clear (approx. 2 to 2 1/2 minutes, depending on the microwave). The agarose is cooled to the point that the glassware can be handled, but the agarose is still fluid. Add the EtBr at this point in the quantity of 0.2 µL EtBr per 1 mL agarose. The agarose is poured onto the gel tray and allowed to solidify.

3. To the solidified gel, add the 1X TBE with EtBr as tank buffer, filling the gel box until the gel is covered.

4. Using a filtered micropipette tip, mix the reaction product (from **Section 4.2**) with 10X BlueJuice or equivalent loading buffer at a ratio of 10:1 (reaction product to BlueJuice) in a sterile tube or 96-well microtiter plate. For example, a well that would contain 20 µL would have 18 µL of reaction product mixed with 2 µL of loading buffer. Mix by pipetting up and down several times. Repeat step for all samples. Make sure to change tips for each sample.

5. Pipette all of the loading buffer product mixture into the respective gel well for that sample, reserving the first lane for the 100 bp ladder. Load all samples, making sure to change tips for each sample. Record the order that the samples are loaded on the current version of the lane worksheet **VIRTWS0419**.

6. Pipette the 100 bp ladder to the first lane of the gel and add to last lane of gel if needed. Record the addition of the ladder on the lane worksheet. The ladder is added last because some ladders have a tendency to float out of the well. Normally the volume of ladder added is the established volume of the well, but if the ladder does float easily, a volume of ladder less then that of the volume of the well can be used. For instance, for a gel with a 20 µL well volume, 10 or 15 µL of ladder could be added if floating is a concern.

4.3.2 Running gel

1. Replace the electrophoresis apparatus cover, matching black and red connectors. Attach electrodes with the appropriate positive (red) and negative (black) leads to the power supply. Verify that the entire apparatus is level. The current runs negative to positive from the top of gel.

2. Set the power supply to run at an appropriate voltage. This varies with the type of gel box used and the concentration of the gel. What is usually used is 100 volts for 60 to 80 minutes for a 2% TBE gel.

3. Power up the unit and apply current to the gel. Observe that the apparatus is operating correctly by the formation of bubbles along the black electrode of the apparatus. If bubbles do not form, the electrophoresis apparatus is not working right. Make sure the black electrode is at the top, that all connections are connected properly, and that the gel box is on and is working properly. Allow the current to run until dye has migrated close to the bottom edge of the gel. **Do not let the dye run off the gel.**

4. Turn the power supply off.

4.3.3 Visualizing and documenting gel

1. Carefully disassemble the gel apparatus. Refer to **2.2.13** for safe handling of gels containing EtBr. Lift the tray holding the gel out of the apparatus.

2. Place the tray in a shallow container or on several sheets of Kimwipes if not moving it very far, and transport gel to UV light box.

3. View and photograph gel according to available programs. The current program for recording pictures is the Kodak Gel Logic 2200 imaging system and the analysis program is Kodak Molecular Imaging Software version 4.0.4 or current version image analysis software. Attach a photograph or electronic picture to the current version of **VIRTWS0519** and retain the original copy with the batch records. After photographing, dispose of gel in a container designated for solid EtBr waste. Empty the gel buffer containing EtBr into a designated container. **DO NOT pour EtBr buffer down the sink.** After disposing of the buffer, rinse the gel box and associated parts (gel tray, comb, lid, etc.) with tap water and allow to air dry.

5. Interpretation of the Test Results

Compare any visible bands to the standard ladders. REV has a product size of **291 bp** when compared to the bands on the 100 bp ladder.

Test criteria are:

1. Internal REV positive controls have visible bands at 291 base pairs.
2. Sample only lanes having no visible bands at 291 bp.
3. External negative control has no visible band at 291 bp.
4. External positive control has a visible band at 291 bp.

If all four criteria are met, testing is considered satisfactory and samples negative for extraneous REV. If criteria #1 or #3 are not met, testing is considered invalid. If criterion #4 is not met, testing may still be considered valid and satisfactory and negative for extraneous REV. If criterion #2 is not met, testing is considered unsatisfactory.

Invalid testing will be repeated.

At supervisory discretion, unsatisfactory testing will be repeated until either 2 consecutive tests are satisfactory and negative for extraneous REV or until 2 consecutive tests are unsatisfactory and positive for extraneous REV.

6. Report of Test Results

Report results on report out worksheet **VIRTWS0519.** Results are reviewed and entered into proper computer databases according to the current version of **VIRSOP0027**.

7. Summary of Revisions

- **2.2.4:** "Six Paq" has been removed and the part number changed.
- **2.2.10:** Track-It and Invitrogen have been removed and replaced with PCRSizer and Norgen. The catalog number has been changed.
- **3.3.3:** "Once thawed, refrigerate" has been added.
- **3.3.4:** 100 µL has been removed and replaced with 200 µL. "And a negative cell control" has been added.
- **4.1:** The process has been clarified.
- **4.2.2:** "Water" has been removed and replaced with "Negative".
- **4.2.6:** "Extracted negative cell control" has been added.

- **4.3.1:** The steps have been restructured for clarification. The number of steps has been changed.

- **4.4.3:** "Or on several sheets of Kimwipes if not moving the tray very far" has been added.